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Improved medium for large-scale production of recombinant cholera toxin B subunit for vaccine purposes

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Abstract

Cholera toxin B subunit (CTB) is an important component of the new oral cholera vaccine. CTB can also be used as a potential carrier protein for foreign antigens or epitopes covalently linked to this molecule, such as carbohydrates or peptides. In order to obtain large amounts of CTB, high-level expressions, which facilitate its production, have been introduced. We were able to develop a plasmid expression system that, when used in conjunction with an optimized culture medium, provided yield of CTB more than 800 mg per liter. Based on syncase medium, the higher levels of CTB production were obtained by raising the concentration of casamino acids twice and reduction of the sucrose level down to 50% of the original formula. This simple change resulted in 3-fold increasement of CTB expression into the medium. Using our method, production and purification of cholera toxin B subunit become simple, inexpensive and very effective.

Keywords: Cholera toxin B subunit; *Vibrio cholerae*; Large-scale production; Medium

1. Introduction

Infection with *Vibrio cholerae* O1 strains is a significant problem in many developing countries. *V. cholerae* causes diarrhea through elaboration of cholera toxin (CT) which is directly responsible for characteristic electrolyte and fluid secretion from the small intestine [1]. Cholera toxin consists of a single A subunit (CTA) responsible for the activation of adenylate cyclase in the intestinal cells and five B subunits (CTB) that bind the holotoxin to GM1 ganglioside receptors. The B subunit of CT is non-toxic and has been shown to possess high immunogenic activity [2].

The ability of CTB to elicit a mucosal immune response makes it an attractive candidate as a vaccine component. It has been demonstrated in a large randomized placebo-controlled field trial that the oral cholera vaccine containing the B subunit of CT together with inactivated *V. cholerae* cells had the advantage over the whole-cell vaccine alone of a significantly higher efficacy level for the initial 4–6 months period [3].

Because of its binding ability to intestinal epithelial cells, CTB can also be used as an immunogenic carrier for peptide and carbohydrate antigens. Such antigens coupled to CTB either chemically or genetically fused could be administered orally and give rise to local and systemic immune responses [4–6].

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Cholera toxin B subunit also has adjuvant properties and when administered together with less potent antigens resulted in a significant enhancement of the antibody response [4,7].

In order to obtain large amounts of cholera toxin B subunit, over-expression systems for CTB have been described [8–10]. With this approach it is possible to establish large-scale production of recombinant CTB which is essentially identical to the native product. However, it is also important to determine optimal culture conditions for expression of such cholera toxin B subunit. In this paper we describe a modified syncase medium which facilitates large-scale production of cholera toxin B subunit by *V. cholerae* CTB recombinant strains, e.g. for vaccine development purposes.

2. Materials and methods

2.1. Bacterial strains

The following *Vibrio cholerae* strains were used: 569B, classical Inaba; JS1569(pJS752-3), a rifampicin-resistant, *ctx*A-deleted derivative of 569B [9], and JS1569(pML-LCTB*tac*2) which harbours a high copy number plasmid with the *ctx*B gene placed under the control of the strong *tac* promoter [8]. The construction of the *V. cholerae* recombinant stains is described elsewhere. Briefly, in the strain JS1569(pJS752-3) the structural gene encoding the CTB was placed under the control of the strong *tac*P promoter in a wide host range plasmid [9]; this construct produced a reproducibly high level of CTB. To further improve productivity of the recombinant toxin, the copy number plasmid was increased by the addition of the origin of replication from plasmid pUC19 [8]. This new plasmid (pML-LCTB*tac*2) allows the accumulation of a homogeneous recombinant CTB that is essentially identical to the native product and which secreted more than 95% to the culture medium [8]. The strains were stored at −70°C in broth containing 20% glycerol and maintained either on blood agar (strain 569B) or on LB agar plates supplemented with ampicillin, 100 $\mu g\,ml^{-1}$ (the CTB recombinant strains).

2.2. Media

In initial studies, syncase medium described by Finkelstein et al. [11] was used. Based on this medium, several modified media were prepared and tested for CTB production. Preliminary experiments were done with media containing each syncase medium component concentrated twice (syncase 2X) or three times (syncase 3X). Based on the results obtained using these media further modification of the original syncase medium was made. The best results, in terms of CTB production, were achieved with a medium, the composition of which is shown in Table 1. For comparison, the original syncase formula described by Finkelstein et al. [11] is also shown. To prepare the medium, the components were dissolved separately in deionized H_2O, mixed together and autoclaved at 121°C for 20 min. The pH of the medium was 7.8 (adjusted with 1 M HCl, if necessary).

2.3. Culture conditions

Cultures were performed with vigorous shaking at either 30°C or 37°C for periods of 24 h or 48 h, in 250 ml flasks containing 15 ml medium and were analyzed for the optical density (OD) at 600 nm with a Shimadzu double-beam spectrophotometer (Kyoto, Japan) and CTB production (see below).

For large-scale production of CTB, a 5 l bench-top fermentor (New Brunswick Scientific, Edison, NJ, USA) was used. Fermentation was

Table 1
Composition of syncase and modified syncase media ($g\,l^{-1}$)

	Syncase*	Modified Syncase
Casamino acids	10.0	20.0
Sucrose	5.0	2.5
NH_4Cl	1.18	1.18
Na_2SO_4	0.089	0.089
$Na_2HPO_4.2H_2O$	6.27	6.27
$K_2HPO_4.3H_2O$	6.55	6.55
$MgCl_2.6H_2O$	0.042	0.042
$MnCl_2.4H_2O$	0.004	0.004
$FeCl_3.6H_2O$	0.005	0.005

* Medium described by Finkelstein et al. [11].

performed for 24 h at 37°C in 3 l medium with high aeration (air flow 5 l min^{-1}) and revolution 500 r.p.m. Fermentors were inoculated with an overnight culture grown at 37°C in four 250 ml flasks with 40 ml medium. During fermentation, 5 ml samples were taken and pH, OD 600, and CTB, respectively, were determined.

2.4. CTB determination

The concentration of cholera toxin B subunit was determined by GM1 ELISA with monoclonal antibodies specific to CTB as described previously [12,13] or by the single radial immunodiffusion test [14] with rabbit antisera raised against highly purified homologous CTB of *V. cholerae* strains JS1569(pJS752-3) and JS1569(pML-LCTB*tac*2), respectively. For both analyses, one ml of the culture samples were collected, centrifuged (12,000*g*, 5 min) and the supernatants were tests for CTB.

3. Results

3.1. Development of the modified syncase medium

The improved medium was developed to increase large-scale production of cholera toxin B subunit, primarily for vaccine purposes. In preliminary tests several commonly used media (Syncase; LB; Brain Heart Infusion; Minimal M9; Peptone Water; Casamino Acids–Yeast Extract) were tested for CTB production. The results obtained led to the conclusion that syncase was the best medium in terms of the yield of the CTB produced by both 569B and the *ctx* recombinant *V. cholerae* strains (data not shown). Based on these results further experiments were performed to compare CTB production by *V. cholerae* strains in original syncase and media which were modified by increasing the concentration of each syncase component to two (syncase 2X) or to three times normal (syncase 3X). It was noted (Table 2) that higher amounts of CTB were produced by strain 569B grown at 30°C in syncase as compared to concentrated media. On the contrary, better results in terms of CTB production by the recombinant strains JS1569(pJS752-3) and JS1569(pML-LCTB*tac*2) were achieved when cultures were performed at 37°C in syncase 2X (Table 2). It was observed that further increase of medium concentration (up to 3 times, syncase 3X) suppressed CTB production at both 30°C and 37°C, for both the wild type and recombinant strains. Based on these data further improvement of the syncase 2X medium was performed. The optimal concentration of each component was evaluated

Table 2
CTB production ($\mu g\ ml^{-1}$) by *V. cholerae* strains grown in syncase and concentrated syncase media

Medium	Temperature	*V. cholerae* strains		
		569B	JS1569(pJS752-3)	JS1569(pML-LCTB*tac*2)
Syncase	30°C	21.9 ± 4.5[a]	59 ± 4	155 ± 12
	37°C	0.5 ± 0.1	113 ± 16	192 ± 82
Syncase 2X	30°C	5.4 ± 0.7	190 ± 27	236 ± 58
	37°C	1.6 ± 0.3	242 ± 27	316 ± 99
Syncase 3X	30°C	1.0 ± 0.2	107 ± 7	162 ± 17
	37°C	0.3 ± 0.1	118 ± 26	185 ± 10

[a] CTB was determined in supernatants of 24 h shaking flask cultures using GM1 ELISA or single radial immunodiffusion tests; results are based on three separate experiments; the values represent geometric mean ± standard deviation.

using a checkerboard test. It was noted that higher levels of CTB expression are obtained by raising the concentration of casamino acids in the presence of the same level of the other components as described for the original syncase and further evaluation of the sucrose level resulted in a medium which improved CTB production further. The final modified medium contained twice the concentration of casamino acids supplied in the original syncase formula and half the amount of sucrose (Table 1). This medium gave a very high CTB productivity by both *V. cholerae* recombinant strains especially pronounced for the strain JS1569(pML-LCTB*tac*2). Interestingly, this effect was not observed when strain 569B was tested (Table 3).

3.2. Temperature dependent of CTB expression

Regarding the temperature, 37°C was better than 30°C for CTB production by strains JS1569(pJS752-3) and JS1569(pML-LCTB*tac*2), both in syncase and modified syncase media (Table 3). The average amount of CTB produced by strain JS1569(pML-LCTB*tac*2) at 37°C in modified syncase medium after 24 h and 48 h was $590 \pm 88\ \mu g\ ml^{-1}$ and $837 \pm 64\ \mu g\ ml^{-1}$, respectively. When the strain was cultured in syncase medium using the same conditions, the levels of CTB were only $192 \pm 82\ \mu g\ ml^{-1}$ (24 h culture) and $290 \pm 20\ \mu g\ ml^{-1}$ (48 h culture).

To compare CTB production by *V. cholerae* strains tested in the modified syncase and original medium, ratios between the levels of CTB in both media tested under the same conditions were calculated (Table 3). It was noted that strain 569B grown at 30°C in original syncase produced more CTB than when grown in the modified medium, both in 24 h and 48 h cultures (ratio 0.66 and 0.78, respectively). Toxins levels were slightly higher in the new medium when cultures of strain 569B were performed at 37°C (ratio 1.20).

The advantage of the modified syncase medium over original syncase was most clearly pronounced for the CTB recombinant *V. cholerae* strains. It was shown that strains JS1569(pJS752-3) and JS1569(pML-LCTB*tac*2) produced more toxin when cultured in modified medium, both at 30°C and 37°C. The best results were achieved when strain JS1569(pML-LCTB*tac*2) was grown at 37°C. After 24 h the ratio of CTB levels in modified syncase as compared to the original medium was 3.07, i.e. the level of CTB in the new medium was threefold higher as compared to that achieved from the same strain grown in syncase. Therefore, temperature 37°C and strain JS1569(pML-

Table 3
CTB production ($\mu g\ ml^{-1}$) by *V. cholerae* strains grown in the modified syncase medium[a] and (in parentheses) ratios of the CTB levels in modified syncase as compared to syncase medium

V. cholerae strain	Culture conditions			
	30°C		37°C	
	24 h	48 h	24 h	48 h
569B	14.4 ± 2.6[b] (0.66)[c]	18 ± 0.9 (0.78)	0.6 ± 0.2 (1.20)	0.6 ± 0.1 (1.20)
JS1569(pJS752-3)	95 ± 7 (1.61)	116 ± 12 (1.61)	186 ± 6 (1.65)	213 ± 16 (1.44)
JS1569(pML-LCTB*tac*2)	312 ± 6 (2.01)	378 ± 88 (1.73)	590 ± 88 (3.07)	837 ± 64 (2.89)

[a] Composition of the medium is shown in Table 1.
[b] CTB was determined in supernatants of shaking flask cultures using GM1 ELISA or single radial immunodiffusion tests. Results are based on three separate experiments. The values represent geometric mean ± standard deviation.
[c] Ratios between geometric mean of CTB levels produced by the *V. cholerae* strains determined in supernatants of shaking flask cultures in modified syncase and syncase media. Results are based on three separate experiments.

LCTB*tac*2) were used in subsequent cultures in a bench-top fermentor.

3.3. Kinetics of CTB production in a fermentor

During fermentation performed for 24 h in syncase medium, OD 600 reached its maximum at 7.46 after 9 h. When the modified syncase medium was used the growth kinetic was similar (Fig. 1A), however, the maximum OD was 9.0 (8 h culture). The pH of 7.8 at the start of the fermentations decreased during the first 4 h and reached its minimum of 6.89 (syncase) and 7.27 (modified syncase), respectively. After that, the pH values of both cultures showed a slow constant increase and pH reached 8.67 and 9.22 in syncase and modified syncase respectively, at the end of the fermentation process (24 h) (Fig. 1B). Cholera toxin B subunit accumulation patterns are shown in Fig. 1C. It can be seen that there was a correlation between growth and CTB expression. The majority of the product accumulated during late logarithmic and stationary phases when the sucrose carbon source has been exhausted. During fermentation performed in syncase the concentration of CTB did not exceed 200 mg l^{-1}. Using modified syncase medium the amount of CTB obtained was over 600 mg l^{-1}, i.e., a three-fold higher level than obtained under the same culture conditions in original syncase medium.

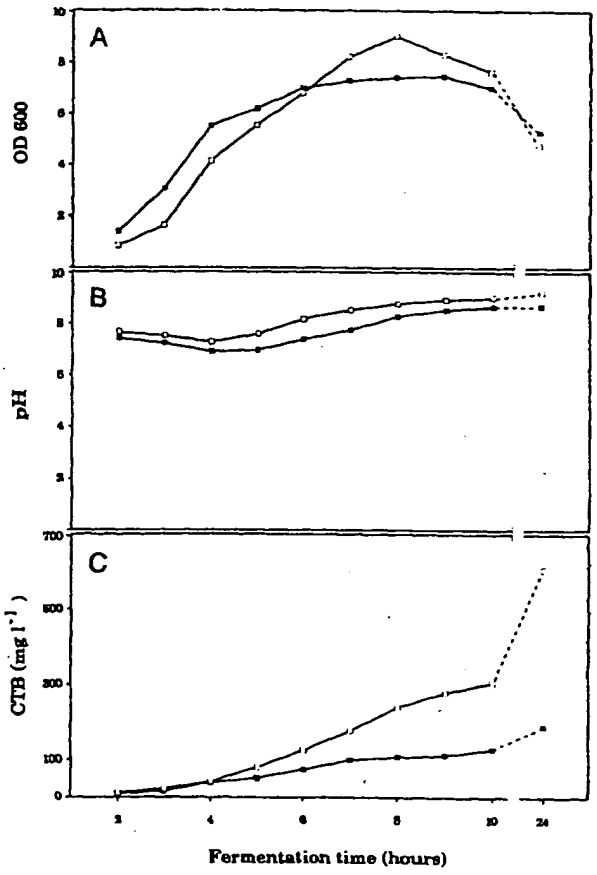

Fig. 1. The kinetics of growth (A), pH (B), and CTB production (C) of *V. cholerae* strain JS1569(pML-LCTB*tac*2) during fermentations performed in a 5-1 fermentor in syncase (—■—) and modified syncase (—□—) media, respectively.

4. Discussion

Many efforts have been made to increase production of cholera toxin B subunit by *V. cholerae ctx* recombinant strains. Different overexpression systems have been established and described [8–10]. Recombinant *V. cholerae* strains carrying *ctx*B genes produce large amounts of CTB which are fully secreted to the medium from where they can be simply purified.

From the practical point of view, optimization of the culture conditions used for such *V. cholerae* strains is also important. In this report a modified syncase medium of Finkelstein et al. [11] is described.

We found that production of CTB by both our *V. cholerae* recombinant strains (JS1569(pJS752-3) and JS1569(pML-LCTB*tac*2)) significantly depended on the concentration of the medium components and incubation temperature. It was found, using a checker-board titration, that the most important roles for stimulating CTB production were played by casamino acids and sucrose concentration. Therefore, the final medium contained as much as twice the amount of casamino acids and half the amount of sucrose as compared to the original syncase formula. The casamino acids as a nitrogen source seem to be especially important. Indeed, when their concentration was reduced to 15 g l^{-1} (with the same amount of other components described for the modified medium) it resulted in a decrease in

CTB production (data not shown). Also, the replacement of the casamino acids with another carbon source (peptone) did not result in significantly higher toxin levels (data not shown).

In the new medium the CTB production by both *V. cholerae ctx* recombinant strains dramatically increased. However, this effect was not observed for the parent wild type strain 569B which produced more cholera toxin in syncase as compared with modified syncase medium. The explanation for this remains unclear.

The temperature of incubation also had significant influence on the production of the recombinant CTB. The strains elaborated more cholera toxin B subunit when grown at 37°C than at 30°C. This effect was noted both for syncase and modified syncase media. However, the influence of the higher temperature was more clearly pronounced for the strain JS1569(pML-LCTB*tac*2) cultured in the new medium; in this condition the strain produced 3 times more CTB than under the same culture conditions in original syncase (Table 3). These data concerning the growth temperature are opposite to the results described by Van de Walle et al. [15] which found that a temperature of 30°C rather than 37°C was optimal for CTB production by *ctx*A deleted strain *V. cholerae* O395-NI. On the other hand, we have also noted that the wild-type strain 569B produced more toxin when cultured at 30°C, in both media tested (i.e. syncase and modified syncase, respectively). Indeed, the production of cholera toxin by *V. cholerae* strains is coordinately regulated by the ToxR system [16]. The *tox*R gene has been shown to be a transcriptional activator of the cholera toxin operon [17] and the ToxR exhibits its maximal activity at subphysiological temperatures, i.e. at 30°C rather than 37°C.

Production of the recombinant CTB by both strains used in the present study was correlated with bacterial growth; however, there was the maximal toxin accumulation during the stationary phase. This pattern was clearly seen when the strain JS1569(pML-LCTB*tac*2) was cultured in the modified medium. However, it was observed that CTB accumulation did not simply depend on the bacterial cell concentration: the maximum optical density of the cells measured in the modified medium was not significantly higher as compared with OD 600 of the original syncase culture.

The *tac* promoter-based vectors used in our *V. cholerae* recombinant strains have been demonstrated, under favourable growth conditions described, to allow the accumulation of large amounts of CTB in the growth medium. It was found that the highest toxin level expressions were obtained when the strain JS1569(pML-CTB*tac*2) harbouring a high copy number plasmid was grown in the medium containing less carbohydrate carbon source and more casamino acids. This higher amino acid concentration supplied in casamino acids may be one explanation of that effective CTB secretivity. As we tested, following growth to the stationary phase, the *V. cholerae* cells did not lose their capacity to produce CTB. Cultures were harvested and resuspended in an equal volume of fresh syncase medium. In the absence of significant growth, CTB continued accumulation to concentrations equivalent to those found in the cultures performed in our modified syncase medium which contained more amino acids from the beginning. Also, the percentage of plasmid-bearing cells prior and after induction in both media tested were similar (data not shown).

It also seems that a marked enhancement in CTB production in our modified medium reflects a higher efficiency in the secretion process of the recombinant protein. The possible role, if any, of released proteases seems less important. The levels of the soluble hemagglutinin/protease (SHA) were similar in cultures grown in both media as tested in ELISA [18] (data not shown). However, one cannot totally exclude a proteolytic role of other proteases secreted by *V. cholerae* strains grown in syncase, although this explanation of the lower toxin levels obtained in this medium seems less likely.

In conclusion, we have modified syncase medium to optimize the production of the recombinant cholera toxin B subunit for use as a protective immunogen of the oral cholera vaccine [19] and for other purposes, e.g. as a biological carrier of heterologous antigens (epi-

topes) or as a mucosal adjuvant [4]. For these purposes a large amount of the recombinant cholera toxin B subunit is needed. This can be achieved both by the use of CTB over expression systems and optimal culture conditions described in this report. Our findings can also greatly simplify large-scale production of the currently used oral B subunit-whole cell vaccine against cholera, reduce its cost and allow local preparation of the toxin component of this vaccine in developing countries.

Acknowledgements

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1. Introduction

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The ability of CTB to elicit a mucosal immune response makes it an attractive candidate as a vaccine component. It has been demonstrated in a large randomized placebo-controlled field trial that the oral cholera vaccine containing the B subunit of CT together with inactivated *V. cholerae* cells had the advantage over the whole-cell vaccine alone of a significantly higher efficacy level for the initial 4–6 months period [3].

Because of its binding ability to intestinal epithelial cells, CTB can also be used as an immunogenic carrier for peptide and carbohydrate antigens. Such antigens coupled to CTB either chemically or genetically fused could be administered orally and give rise to local and systemic immune responses [4–6].

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2.2. Media

In initial studies, syncase medium described by Finkelstein et al. [11] was used. Based on this medium, several modified media were prepared and tested for CTB production. Preliminary experiments were done with media containing each syncase medium component concentrated twice (syncase 2X) or three times (syncase 3X). Based on the results obtained using these media further modification of the original syncase medium was made. The best results, in terms of CTB production, were achieved with a medium, the composition of which is shown in Table 1. For comparison, the original syncase formula described by Finkelstein et al. [11] is also shown. To prepare the medium, the components were dissolved separately in deionized H_2O, mixed together and autoclaved at 121°C for 20 min. The pH of the medium was 7.8 (adjusted with 1 M HCl, if necessary).

2.3. Culture conditions

Cultures were performed with vigorous shaking at either 30°C or 37°C for periods of 24 h or 48 h, in 250 ml flasks containing 15 ml medium and were analyzed for the optical density (OD) at 600 nm with a Shimadzu double-beam spectrophotometer (Kyoto, Japan) and CTB production (see below).

For large-scale production of CTB, a 5 l bench-top fermentor (New Brunswick Scientific, Edison, NJ, USA) was used. Fermentation was

Table 1
Composition of syncase and modified syncase media ($g\,l^{-1}$)

	Syncase*	Modified Syncase
Casamino acids	10.0	20.0
Sucrose	5.0	2.5
NH_4Cl	1.18	1.18
Na_2SO_4	0.089	0.089
$Na_2HPO_4.2H_2O$	6.27	6.27
$K_2HPO_4.3H_2O$	6.55	6.55
$MgCl_2.6H_2O$	0.042	0.042
$MnCl_2.4H_2O$	0.004	0.004
$FeCl_3.6H_2O$	0.005	0.005

* Medium described by Finkelstein et al. [11].

performed for 24 h at 37°C in 3 l medium with high aeration (air flow 5 l min^{-1}) and revolution 500 r.p.m. Fermentors were inoculated with an overnight culture grown at 37°C in four 250 ml flasks with 40 ml medium. During fermentation, 5 ml samples were taken and pH, OD 600, and CTB, respectively, were determined.

2.4. CTB determination

The concentration of cholera toxin B subunit was determined by GM1 ELISA with monoclonal antibodies specific to CTB as described previously [12,13] or by the single radial immunodiffusion test [14] with rabbit antisera raised against highly purified homologous CTB of *V. cholerae* strains JS1569(pJS752-3) and JS1569(pML-LCTB*tac*2), respectively. For both analyses, one ml of the culture samples were collected, centrifuged (12,000*g*, 5 min) and the supernatants were tests for CTB.

3. Results

3.1. Development of the modified syncase medium

The improved medium was developed to increase large-scale production of cholera toxin B subunit, primarily for vaccine purposes. In preliminary tests several commonly used media (Syncase; LB; Brain Heart Infusion; Minimal M9; Peptone Water; Casamino Acids–Yeast Extract) were tested for CTB production. The results obtained led to the conclusion that syncase was the best medium in terms of the yield of the CTB produced by both 569B and the *ctx* recombinant *V. cholerae* strains (data not shown). Based on these results further experiments were performed to compare CTB production by *V. cholerae* strains in original syncase and media which were modified by increasing the concentration of each syncase component to two (syncase 2X) or to three times normal (syncase 3X). It was noted (Table 2) that higher amounts of CTB were produced by strain 569B grown at 30°C in syncase as compared to concentrated media. On the contrary, better results in terms of CTB production by the recombinant strains JS1569(pJS752-3) and JS1569(pML-LCTB*tac*2) were achieved when cultures were performed at 37°C in syncase 2X (Table 2). It was observed that further increase of medium concentration (up to 3 times, syncase 3X) suppressed CTB production at both 30°C and 37°C, for both the wild type and recombinant strains. Based on these data further improvement of the syncase 2X medium was performed. The optimal concentration of each component was evaluated

Table 2
CTB production ($\mu g\ ml^{-1}$) by *V. cholerae* strains grown in syncase and concentrated syncase media

Medium	Temperature	*V. cholerae* strains		
		569B	JS1569(pJS752-3)	JS1569(pML-LCTB*tac*2)
Syncase	30°C	21.9 ± 4.5[a]	59 ± 4	155 ± 12
	37°C	0.5 ± 0.1	113 ± 16	192 ± 82
Syncase 2X	30°C	5.4 ± 0.7	190 ± 27	236 ± 58
	37°C	1.6 ± 0.3	242 ± 27	316 ± 99
Syncase 3X	30°C	1.0 ± 0.2	107 ± 7	162 ± 17
	37°C	0.3 ± 0.1	118 ± 26	185 ± 10

[a] CTB was determined in supernatants of 24 h shaking flask cultures using GM1 ELISA or single radial immunodiffusion tests; results are based on three separate experiments; the values represent geometric mean ± standard deviation.

using a checkerboard test. It was noted that higher levels of CTB expression are obtained by raising the concentration of casamino acids in the presence of the same level of the other components as described for the original syncase and further evaluation of the sucrose level resulted in a medium which improved CTB production further. The final modified medium contained twice the concentration of casamino acids supplied in the original syncase formula and half the amount of sucrose (Table 1). This medium gave a very high CTB productivity by both *V. cholerae* recombinant strains especially pronounced for the strain JS1569(pML-LCTB*tac*2). Interestingly, this effect was not observed when strain 569B was tested (Table 3).

3.2. Temperature dependent of CTB expression

Regarding the temperature, 37°C was better than 30°C for CTB production by strains JS1569(pJS752-3) and JS1569(pML-LCTB*tac*2), both in syncase and modified syncase media (Table 3). The average amount of CTB produced by strain JS1569(pML-LCTB*tac*2) at 37°C in modified syncase medium after 24 h and 48 h was $590 \pm 88\ \mu g\ ml^{-1}$ and $837 \pm 64\ \mu g\ ml^{-1}$, respectively. When the strain was cultured in syncase medium using the same conditions, the levels of CTB were only $192 \pm 82\ \mu g\ ml^{-1}$ (24 h culture) and $290 \pm 20\ \mu g\ ml^{-1}$ (48 h culture).

To compare CTB production by *V. cholerae* strains tested in the modified syncase and original medium, ratios between the levels of CTB in both media tested under the same conditions were calculated (Table 3). It was noted that strain 569B grown at 30°C in original syncase produced more CTB than when grown in the modified medium, both in 24 h and 48 h cultures (ratio 0.66 and 0.78, respectively). Toxins levels were slightly higher in the new medium when cultures of strain 569B were performed at 37°C (ratio 1.20).

The advantage of the modified syncase medium over original syncase was most clearly pronounced for the CTB recombinant *V. cholerae* strains. It was shown that strains JS1569(pJS752-3) and JS1569(pML-LCTB*tac*2) produced more toxin when cultured in modified medium, both at 30°C and 37°C. The best results were achieved when strain JS1569(pML-LCTB*tac*2) was grown at 37°C. After 24 h the ratio of CTB levels in modified syncase as compared to the original medium was 3.07, i.e. the level of CTB in the new medium was threefold higher as compared to that achieved from the same strain grown in syncase. Therefore, temperature 37°C and strain JS1569(pML-

Table 3
CTB production ($\mu g\ ml^{-1}$) by *V. cholerae* strains grown in the modified syncase medium[a] and (in parentheses) ratios of the CTB levels in modified syncase as compared to syncase medium

V. cholerae strain	Culture conditions			
	30°C		37°C	
	24 h	48 h	24 h	48 h
569B	14.4 ± 2.6[b] (0.66)[c]	18 ± 0.9 (0.78)	0.6 ± 0.2 (1.20)	0.6 ± 0.1 (1.20)
JS1569(pJS752-3)	95 ± 7 (1.61)	116 ± 12 (1.61)	186 ± 6 (1.65)	213 ± 16 (1.44)
JS1569(pML-LCTB*tac*2)	312 ± 6 (2.01)	378 ± 88 (1.73)	590 ± 88 (3.07)	837 ± 64 (2.89)

[a] Composition of the medium is shown in Table 1.
[b] CTB was determined in supernatants of shaking flask cultures using GM1 ELISA or single radial immunodiffusion tests. Results are based on three separate experiments. The values represent geometric mean ± standard deviation.
[c] Ratios between geometric mean of CTB levels produced by the *V. cholerae* strains determined in supernatants of shaking flask cultures in modified syncase and syncase media. Results are based on three separate experiments.

LCTB*tac*2) were used in subsequent cultures in a bench-top fermentor.

3.3. Kinetics of CTB production in a fermentor

During fermentation performed for 24 h in syncase medium, OD 600 reached its maximum at 7.46 after 9 h. When the modified syncase medium was used the growth kinetic was similar (Fig. 1A), however, the maximum OD was 9.0 (8 h culture). The pH of 7.8 at the start of the fermentations decreased during the first 4 h and reached its minimum of 6.89 (syncase) and 7.27 (modified syncase), respectively. After that, the pH values of both cultures showed a slow constant increase and pH reached 8.67 and 9.22 in syncase and modified syncase respectively, at the end of the fermentation process (24 h) (Fig. 1B). Cholera toxin B subunit accumulation patterns are shown in Fig. 1C. It can be seen that there was a correlation between growth and CTB expression. The majority of the product accumulated during late logarithmic and stationary phases when the sucrose carbon source has been exhausted. During fermentation performed in syncase the concentration of CTB did not exceed 200 mg l^{-1}. Using modified syncase medium the amount of CTB obtained was over 600 mg l^{-1}, i.e., a three-fold higher level than obtained under the same culture conditions in original syncase medium.

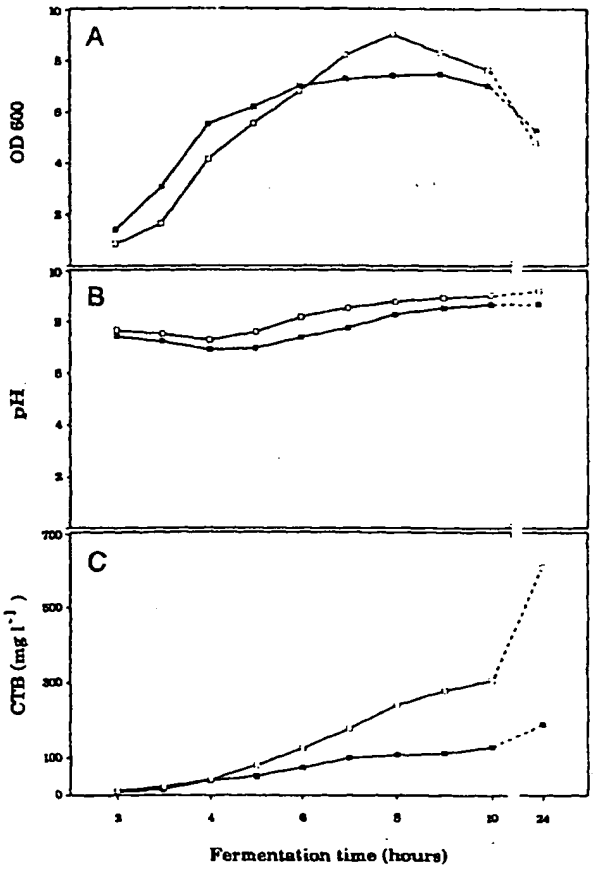

Fig. 1. The kinetics of growth (A), pH (B), and CTB production (C) of *V. cholerae* strain JS1569(pML-LCTB*tac*2) during fermentations performed in a 5-l fermentor in syncase (—■—) and modified syncase (—□—) media, respectively.

4. Discussion

Many efforts have been made to increase production of cholera toxin B subunit by *V. cholerae ctx* recombinant strains. Different overexpression systems have been established and described [8–10]. Recombinant *V. cholerae* strains carrying *ctx*B genes produce large amounts of CTB which are fully secreted to the medium from where they can be simply purified.

From the practical point of view, optimization of the culture conditions used for such *V. cholerae* strains is also important. In this report a modified syncase medium of Finkelstein et al. [11] is described.

We found that production of CTB by both our *V. cholerae* recombinant strains (JS1569(pJS752-3) and JS1569(pML-LCTB*tac*2)) significantly depended on the concentration of the medium components and incubation temperature. It was found, using a checker-board titration, that the most important roles for stimulating CTB production were played by casamino acids and sucrose concentration. Therefore, the final medium contained as much as twice the amount of casamino acids and half the amount of sucrose as compared to the original syncase formula. The casamino acids as a nitrogen source seem to be especially important. Indeed, when their concentration was reduced to 15 g l^{-1} (with the same amount of other components described for the modified medium) it resulted in a decrease in

CTB production (data not shown). Also, the replacement of the casamino acids with another carbon source (peptone) did not result in significantly higher toxin levels (data not shown).

In the new medium the CTB production by both *V. cholerae ctx* recombinant strains dramatically increased. However, this effect was not observed for the parent wild type strain 569B which produced more cholera toxin in syncase as compared with modified syncase medium. The explanation for this remains unclear.

The temperature of incubation also had significant influence on the production of the recombinant CTB. The strains elaborated more cholera toxin B subunit when grown at 37°C than at 30°C. This effect was noted both for syncase and modified syncase media. However, the influence of the higher temperature was more clearly pronounced for the strain JS1569(pML-LCTB*tac*2) cultured in the new medium; in this condition the strain produced 3 times more CTB than under the same culture conditions in original syncase (Table 3). These data concerning the growth temperature are opposite to the results described by Van de Walle et al. [15] which found that a temperature of 30°C rather than 37°C was optimal for CTB production by *ctx*A deleted strain *V. cholerae* O395-NI. On the other hand, we have also noted that the wild-type strain 569B produced more toxin when cultured at 30°C, in both media tested (i.e. syncase and modified syncase, respectively). Indeed, the production of cholera toxin by *V. cholerae* strains is coordinately regulated by the ToxR system [16]. The *tox*R gene has been shown to be a transcriptional activator of the cholera toxin operon [17] and the ToxR exhibits its maximal activity at subphysiological temperatures, i.e. at 30°C rather than 37°C.

Production of the recombinant CTB by both strains used in the present study was correlated with bacterial growth; however, there was the maximal toxin accumulation during the stationary phase. This pattern was clearly seen when the strain JS1569(pML-LCTB*tac*2) was cultured in the modified medium. However, it was observed that CTB accumulation did not simply depend on the bacterial cell concentration: the maximum optical density of the cells measured in the modified medium was not significantly higher as compared with OD 600 of the original syncase culture.

The *tac* promoter-based vectors used in our *V. cholerae* recombinant strains have been demonstrated, under favourable growth conditions described, to allow the accumulation of large amounts of CTB in the growth medium. It was found that the highest toxin level expressions were obtained when the strain JS1569(pML-CTB*tac*2) harbouring a high copy number plasmid was grown in the medium containing less carbohydrate carbon source and more casamino acids. This higher amino acid concentration supplied in casamino acids may be one explanation of that effective CTB secretivity. As we tested, following growth to the stationary phase, the *V. cholerae* cells did not lose their capacity to produce CTB. Cultures were harvested and resuspended in an equal volume of fresh syncase medium. In the absence of significant growth, CTB continued accumulation to concentrations equivalent to those found in the cultures performed in our modified syncase medium which contained more amino acids from the beginning. Also, the percentage of plasmid-bearing cells prior and after induction in both media tested were similar (data not shown).

It also seems that a marked enhancement in CTB production in our modified medium reflects a higher efficiency in the secretion process of the recombinant protein. The possible role, if any, of released proteases seems less important. The levels of the soluble hemagglutinin/protease (SHA) were similar in cultures grown in both media as tested in ELISA [18] (data not shown). However, one cannot totally exclude a proteolytic role of other proteases secreted by *V. cholerae* strains grown in syncase, although this explanation of the lower toxin levels obtained in this medium seems less likely.

In conclusion, we have modified syncase medium to optimize the production of the recombinant cholera toxin B subunit for use as a protective immunogen of the oral cholera vaccine [19] and for other purposes, e.g. as a biological carrier of heterologous antigens (epi-

topes) or as a mucosal adjuvant [4]. For these purposes a large amount of the recombinant cholera toxin B subunit is needed. This can be achieved both by the use of CTB over expression systems and optimal culture conditions described in this report. Our findings can also greatly simplify large-scale production of the currently used oral B subunit-whole cell vaccine against cholera, reduce its cost and allow local preparation of the toxin component of this vaccine in developing countries.

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